

Distinguishing between anticipatory and responsive plasticity in a seasonally polyphenic butterfly

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Abstract Seasonal generations of short-lived organisms often differ in their morphological, behavioural and life history traits, including body size. These differences may be either due to immediate effects of seasonally variable environment on organisms (responsive plasticity) or rely on presumably adaptive responses of organisms to cues signaling forthcoming seasonal changes (anticipatory plasticity). When directly developing individuals of insects are larger than their overwintering conspecifics, the between-generation differences are typically ascribed to responsive plasticity in larval growth. We tested this hypothesis using the papilionid butterfly *Iphiclides podalirius* as a model species. In laboratory experiments, we demonstrated that seasonal differences in food quality could not explain the observed size difference. Similarly, the size differences are not likely to be explained by the immediate effects of ambient temperature and photoperiod on larval growth. The qualitative pattern of natural size differences between the directly developing and diapausing butterflies could be reproduced in the laboratory as a response to photoperiod, indicating anticipatory character of the response. Directly developing and diapausing individuals followed an identical growth trajectory until the end of the last larval instar, with size differences appearing just a few days before pupation. Taken together, various lines of evidence suggest that between-generation size differences in *I. podalirius* are not caused by immediate effects of environmental factors on larval growth. Instead,

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these differences rather represent anticipatory plasticity and are thus likely to have an adaptive explanation. It remains currently unclear, whether the seasonal differences in adult size *per se* are adaptive, or if they constitute co-product of processes related to the diapause. Our study shows that it may be feasible to distinguish between different types of plasticity on the basis of empirical data even if fitness cannot be directly measured, and contributes to the emerging view about the predominantly adaptive nature of seasonal polyphenisms in insects.

Keywords Seasonal polyphenism · Size dimorphism · Voltinism · Phenology · Lepidoptera

Introduction

Seasonal generations of multivoltine species (i.e. the species with more than one generation per year) often differ considerably in their morphology, behaviour and life history. This type of phenotypic plasticity, called seasonal polyphenism, is common among short-lived organisms like insects and other arthropods, and is particularly well documented in butterflies and moths (reviewed in Shapiro 1976; Tauber et al. 1986; Brakefield 1996; Brakefield and Frankino 2009). For example, different seasonal generations of lepidopterans may differ in morphometrics (Kimura and Masaki 1977; Greene 1989; Brakefield and Larsen 1984; Fric and Konvička 2002; Van Dyck and Wiklund 2002), colour and patterning (Shapiro 1976; Jones 1992; Windig et al. 1994; Hazel et al. 1998; Hazel 2002), body size (references below), growth rate and duration of the larval period (e.g. Wiklund et al. 1991), longevity (Karlsson and Wickman 1989; Brakefield and Frankino 2009) and reproductive traits (Karlsson and Johansson 2008; Larsdotter Mellström et al. 2010).

Phenotypic plasticity is a diverse and complex phenomenon (Pigliucci 2001; West-Eberhard 2003; Whitman and Agrawal 2009). While attempts to classify different responses of the phenotype to the environment have definitely contributed to our understanding, they have also spawned diverse and somewhat inconsistent terminology (Canfield and Greene 2009). One of the proposed classifications discriminates between responsive and anticipatory plasticity (Whitman and Agrawal 2009). Following this framework, *responsive* plasticity (also termed direct plasticity, West-Eberhard 2003) is the result of immediate effects of environmental factors on the organisms. In particular, the impact of nutrition (food quality and quantity) and temperature on life history traits (e.g. Scriber and Slansky 1981; Teder and Tammaru 2005; Kingsolver and Huey 2008) often serve as examples of responsive plasticity. In contrast, in the case of *anticipatory* plasticity (or cued plasticity *sensu* West-Eberhard 2003) phenotypic changes are induced in response to cues which signalize future environmental conditions prior to the actual onset of these conditions. Photoperiod is probably the most widely used cue for seasonal changes, inducing anticipatory responses in various organisms (Nelson et al. 2010). Responsive plasticity needs not to be adaptive (i.e. selected and/or maintained by natural selection) and, indeed, has been sometimes seen as being based on ecological or environmental constraints which prevent an organism from attaining the optimal phenotype under certain environmental conditions (e.g. Blanckenhorn 2009; Borges et al. 2011; Rehan et al. 2011). In contrast, anticipatory plasticity is usually thought to be adaptive. Even if adaptivity cannot perhaps always be uncritically assumed *a priori* (Gotthard and Nylin 1995; Pigliucci 2001; Blanckenhorn 2009), the anticipatory character of a plastic response still provides strong circumstantial evidence of its adaptive basis (Whitman and Agrawal 2009).

Seasonal polyphenisms can typically be classified as anticipatory plasticity, and, indeed, are generally assumed to be adaptive responses to seasonally differing selection pressures (Shapiro 1976; Nijhout 1999, 2003; Brakefield and Frankino 2009). In some cases (e.g. colour polyphenisms in lepidopterans), the adaptive nature of such polyphenisms has explicitly been shown (Kingsolver and Huey 1998; Hazel et al. 1998; Brakefield and Frankino 2009). However, one should not forget that seasonal changes in organismal traits can also result from responsive plasticity as factors typically causing such changes also frequently show clear patterns of seasonal variation. Moreover, as seasonal polyphenisms usually are multitrait responses, it may well be the case that some traits show anticipatory plasticity while differences in others may be a result of responsive plasticity to environmental conditions. Nevertheless, attempts to explicitly confront these different explanations have remained scarce (but see Teder et al. 2010) which is unfortunate given the high potential of seasonal polyphenisms for studies on phenotypic plasticity in general. Indeed, the predictability of seasonally varying selective pressures, many of which are relatively straightforward to understand (Tauber et al. 1986) and even quantify (e.g. Rodrigues and Moreira 2004; Van Asch and Visser 2007; Rimmel et al. 2009) provides an opportunity to analyse the limits to adaptive plastic changes, and the degree to which organisms can evolve to cope with detrimental changes in their environments.

For body size, regular differences between successive generations are reported for various insects, with different species often showing contrasting patterns (Shapiro 1976). For example, in some Lepidoptera the larvae developing early in the season produce larger adults than do late-season larvae (Blau 1981; Tanaka and Tsubaki 1984; Fric and Konvička 2002; Rodrigues and Moreira 2004; current study). By contrast, in some other species, the generation with late-season larvae is the one which attains larger size (Karlssoon and Wickman 1989; Fischer and Fiedler 2001; Liu et al. 2007; Wang et al. 2007; Teder et al. 2010). Several explanations have been proposed to account for these patterns (reviewed in Teder et al. 2010). In particular, higher predation pressure in spring (Rimmel et al. 2009; Teder et al. 2010), higher overwintering survival of larger individuals (Teder et al. 2010) and higher time stress in the directly developing generation (Larsdotter Mellström et al. 2010) have been proposed as the main selective factors to explain size differences between successive generations. However, all these adaptive explanations are applicable only for the case when the late-season larvae grow larger than the early-season larvae.

By contrast, in cases where the insects developing early in the season grow larger, size differences between generations have been typically ascribed to responsive plasticity, i.e. the immediate effects of environmental factors. Indeed, host plant quality (Scriber and Slansky 1981; Schroeder 1986; Van Asch and Visser 2007; Nealis 2012) and sometimes also quantity (Ohgushi 1996; Rodrigues and Moreira 2004) have been shown to decline towards the end of the season. Individuals with their larval period in spring are therefore expected to achieve larger adult sizes for this reason alone (Hahn and Denlinger 2007; Teder et al. 2010). Nevertheless, we are not aware of any studies explicitly exploring the effect of food quality as a mediator of between-generation size differences. Likewise, another candidate for the responsive basis of the between-generation size differences relates to the tendency of ectotherms to grow slower but to mature at larger size when exposed to lower temperatures during the juvenile period (Atkinson 1994; Angilletta and Dunham 2003; Arendt 2011). The generation experiencing lower temperatures during the larval life could thus attain a larger adult size also in response to temperature.

Here we aim at distinguishing between responsive and anticipatory plasticity as the basis for seasonal differences of growth schedules, using the multivoltine papilionid butterfly *Iphiclides podalirius* as a model species. In this species, early-season larvae of the

directly developing generation achieve larger size than late-season larvae of the diapausing generation. First, we use a multiannual dataset on wild-caught individuals to quantify the size-differences between generations in the field. Second, by rearing larvae in controlled conditions at different times of the season, we investigate the role of phenological stage of the host plant leaves (as a proxy of food quality) as a potential determinant of between-generation size differences. Third, by applying different photoperiodic treatments to induce different developmental pathways simultaneously, we study if the size difference is causally linked to the developmental pathway (i.e. direct vs. diapause development) *per se*. Moreover, a detailed comparison of larval growth schedules in the two developmental pathways tells us when and how the size differences appear in the course of larval development. This provides further useful information, and offers a novel way for evaluating the responsive versus anticipatory basis of the between-generation size differences.

Materials and methods

Study species

The scarce swallowtail, *Iphiclides podaricus* L., is a papilionid butterfly with a wide Palaearctic distribution, occurring in Southern and Central Europe as well as in North Africa and in temperate Asia (Tolman and Lewington 2008). The larvae are oligophagous feeding on shrubs and trees of the Rosaceae family, especially on several species of the genus *Prunus* (Stefanescu et al. 2006; Tolman and Lewington 2008). The pupa is the overwintering stage. Depending on the geographic location, this species may have one, two or three generations per year (Tolman and Lewington 2008). In Catalonia (North-East Spain), the area of origin of the individuals used in this study, *I. podalirius* is bivoltine with a partial third generation (Stefanescu et al. 2003; Stefanescu 2004; Table 1). Hereafter these generations are referred to as the *diapausing generation* (larvae in late summer/autumn, overwintering pupae, flight period in spring), the *1st directly developing generation* (larvae in late spring, no pupal diapause, flight period in early summer) and the *2nd directly developing generation* (larvae in early summer, no pupal diapause, flight period in late summer; Table 1).

The scarce swallowtail exhibits seasonal polyphenism in various traits (summarised in Table 1). In addition, the directly developing generation (known also as *I. podalirius* f. *lateri* Austat, 1879) is more abundant than the overwintering generation (f. *miegii* Thierry-Mieg, 1889), the pattern being probably related to the high pupal mortality during the winter diapause (Stefanescu 2004).

Size differences in natural populations

To quantify between-generation size differences in natural populations, 437 adult butterflies representing all three generations were caught in the field in Catalonia over 4 years (1998–2001) and their forewing length was measured. In the analyses, the years were pooled as there were no significant inter-annual differences (Table 2).

Food quality experiment

To investigate the effect of phenological changes in host plant quality on body size, larvae of *I. podalirius* were reared from hatching to pupation at different times of the year (2008).

Table 1 Characteristics of *I. podalirius* generations in the study area (Catalonia, Spain)

Generation	Pupal diapause	Facultative	Preceding generation	Appearance	Flight	Larval period	Prepupal behaviour	Adult abundance
Diapausing	Yes	No	1st or 2nd directly developing	Small adults with darker wings; brown pupae	April and May	July and August or September and October	Long wandering phase; pupation in leaf litter	Medium
1st directly developing	No	No	Diapausing	Large adults with paler wings; green pupae	June and July	May and June	Short wandering phase	High
2nd directly developing	No	Yes	1st directly developing	Large adults with paler wings; green pupae	August or September	June and July	Short wandering phase	Low

Table 2 Results of linear model (based on type 3 sums of squares) for the effects of generation^a and year^b on the adult forewing length in wild-caught sample

Effect	Sexes pooled				Males				Females			
	d.f.	r ² (%)	F	P	d.f.	r ² (%)	F	P	d.f.	r ² (%)	F	P
(G)eneration	1	9.8	149.2	<0.0001	1	19.5	74.6	<0.0001	1	24.8	60.8	<0.0001
(Y)ear	3	0.5	2.3	0.07	3	0.3	0.4	0.8	3	1.5	1.3	0.3
(S)ex	1	19.5	297.0	<0.0001								
G*Y	2	0.02	0.2	0.8	2	0.7	1.3	0.3	2	0.5	0.7	0.5
S*Y	3	0.3	1.5	0.2								
S*G	1	0.7	11.1	0.001								
Error	424	27.8			273	71.2			149	60.8		

See Fig. 1 for sample sizes

^a Diapausing versus directly developing (1st and 2nd directly developing generation pooled) individuals

^b 1998–2001

In particular, “spring trial”, “summer trial” and “autumn trial” were set up so that their timing corresponded to the natural larval periods of the three generations in Catalonia (Table 1). As the quality of tree leaves for lepidopteran herbivores has been reported to decline with progressing season (including *Prunus* species, e.g. Schroeder 1986), host quality was assumed to co-vary with calendar date. Larvae were reared on their most common host plant (*Prunus spinosa*) under controlled conditions at 24 °C, with a photoperiod of 16-h light, 8-h dark (long day conditions) leading to direct development. In all trials, part of the larvae (50, 40 and 90 % in spring, summer and autumn trials, respectively) originated from eggs laid by wild-caught females (a single female in each trial) while the other part hatched from eggs collected in the field.

In all three trials, the larvae were provided leaves from the same three tree individuals. Larvae were kept in Petri dishes (until the last instar, when they were transferred to larger plastic vials) with food being replaced every day. To avoid any position bias, the position of Petri dishes and plastic vials was regularly changed inside the rearing chambers. Larvae were checked daily for moulting, and durations of larval instars were recorded. Pupation date was recorded and pupal weight was determined on the second day after pupation. The insects were sexed as adults.

The experiment was repeated in spring and summer 2009. However, larval mortality was extremely high (close to 90 %) in the summer trial so that weight data from this trial could not be considered in further analyses.

Larval growth experiment

To compare growth curves of the directly developing and diapausing generations, these two developmental pathways were induced simultaneously in the laboratory by applying two photoperiodic treatments. In particular, the “long day treatment” (18-h light, 6-h dark) induced directly developing individuals and the “short day treatment” (12-h light, 12-h dark) gave rise to diapausing individuals. The experiment was performed at the University of Tartu in 2008 and 2009. Larvae hatching on the same day were equally divided between the two photoperiodic treatments and were reared in groups during their first instar. Right

before moulting to their second instar, the larvae were transferred individually to 100 ml plastic vials. The vials were kept on trays in two environmental chambers at 22 °C. All larvae were checked daily and weighed immediately before moulting and on the 2nd and 3rd day of each instar. Pupal weight was determined on the third day after pupation. Diapausing pupae were left to overwinter in environmental chambers at 5 °C for approximately 8 months. Before the emergence of the adults, pupae were transferred to larger plastic boxes to allow the butterflies to spread their wings. Adult weight was determined on the second day after emergence, and forewing length was measured (because of the problems with expanding wings in 2008, only the forewing data from 2009 could be used in the analyses).

In the experiment performed in 2008, we used individuals originating from wild-collected eggs and the progeny of three adult females collected in Catalonia. The larvae hatched at the end of May or in June and were divided between the two photoperiodic treatments, equally with respect to brood and hatching date. Within the environmental chambers (photoperiodic treatments), the position of the vials on the trays was randomized. Larvae were fed with leaves of five closely growing *Prunus domestica* trees in a randomised order. Leaves were renewed every other day. In the experiment performed in 2009 the larvae originated from the eggs laid by 10 artificially paired females (for the artificial pairing technique see Wiklund 1971). Mated females were kept in $0.8 \times 0.8 \times 0.5$ m cages with branches of food plants and a nectar source in the centre of the cage. Such a design (all adults in the same cage) enabled us to obtain considerably more eggs than were available in 2008 at the cost of losing the information on the brood of each larva to be reared.

The experimental protocol of 2009 largely followed the protocol of 2008, with a few changes. Most importantly, last (5th) instar larvae were weighed daily (24 h intervals) to record more details of their growth dynamics. In addition, some methodological changes were introduced. Namely, *Prunus cerasifera* was used as the host plant instead of *P. domestica*; the photoperiodic treatments (and respective larvae) were rotated between the two rearing chambers every 5 days; larvae were transferred to 500 ml plastic boxes after they started to lose weight, and pupae were transferred to 1,000 ml (instead of 500 ml used in 2008) boxes before their emergence.

Data analysis

For forewing length comparisons, a linear model (LM) was fitted with Tukey–Kramer post hoc multiple comparison adjustment for pairwise comparisons. Linear mixed model (LMM) with brood nested within trial as a random factor (SAS, PROC MIXED; Littell et al. 2006) was applied when analysing the data from the food quality experiment. However, as the estimation of degrees of freedom in mixed models with random effects is problematic (Bolker et al. 2009), a Bayesian testing procedure available in the MCMCglmm package for R software (Hadfield 2010) was used in parallel. To compare the sizes and growth trajectories of the directly developing and diapausing generations, linear models were used. However, for the positive growth phase (from moulting to the attaining of the maximal body weight) of the final instar of 2009 experiment, a more detailed analysis was performed by using a non-linear mixed model (R software, nlme package; Pinheiro et al. 2012). In this analysis weight was modelled using a three-parameter logistic function with brood as a random factor and sex, photoperiodic treatment and their interaction as fixed covariates. All analyses were performed with untransformed data as the assumptions of parametric tests were not violated. Unless there were qualitative differences

between the sexes, we present analyses of the data with sexes pooled. Growth rates were calculated as (weight at the end of the period^{1/3}—weight at the beginning of the period^{1/3})/duration of the period (Esperk and Tammaru 2004; Tammaru and Esperk 2007). Calculations were performed separately for the 2nd day of the instar and for the positive growth phase of the instar. Years were pooled in the analyses when the particular experiment was repeated in different years and year*independent variable interaction was not significant.

Results

Adult size (forewing length)

In both wild-caught and laboratory-reared individuals, forewings of directly developing generations were longer than in the diapausing generation (Tables 2, 3; Fig. 1). However, there were no significant size differences between the 1st and 2nd generation directly developing individuals (Fig. 1). The effect of the year on forewing length was not significant in the wild-caught sample (Table 2). Wild-caught individuals were notably larger than those reared in laboratory (Table 3; Fig. 1). The interaction term between generation and origin was significant in the sexes-pooled model (Table 3), indicating higher between-generation size differences in the field than in the rearing experiment.

Food quality experiment

The two methods used to analyze the data of food quality experiment (LMM and Bayesian testing procedure, see “Materials and methods” section) produced both qualitatively and quantitatively similar results. In particular, timing of the experiment (phenological stage of the host plant) had a significant influence on pupal weight in the sexes-pooled model (LMM: $F_{2,47} = 3.3$, $P = 0.047$; MCMC model: $P = 0.009$, $\Delta DIC = 4.2$) and separately in females (LMM: $F_{2,24} = 3.5$, $P = 0.045$; MCMC model: $P = 0.023$, $\Delta DIC = 2.9$), while the relationship was not significant in males (LMM: $F_{2,23} = 1.8$, $P = 0.19$; MCMC model: $P = 0.18$, $\Delta DIC = 1.6$). However, after Tukey–Kramer post hoc multiple

Table 3 Results of linear model (based on type 3 sums of squares) for the effects of generation^a, origin^b on the adult forewing length

Effect	Sexes pooled				Males				Females			
	<i>d.f.</i>	<i>r</i> ² (%)	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>r</i> ² (%)	<i>F</i>	<i>P</i>	<i>d.f.</i>	<i>r</i> ² (%)	<i>F</i>	<i>P</i>
(G)eneration	1	6.3	128.7	<0.0001	1	7.9	48.8	<0.0001	1	30.1	73.0	<0.0001
(O)origin	1	12.1	249.6	<0.0001	1	23.0	142.1	<0.0001	1	20.8	99.1	<0.0001
(S)ex	1	24.1	495.7	<0.0001								
G*O	1	0.3	6.3	0.01	1	0.003	1.8	0.2	1	0.01	4.6	0.03
S*G	1	0.7	14.5	0.0002								
S*O	1	0.06	1.3	0.3								
Error	530	25.8			327	53.0			202	42.5		

See Fig. 1 for sample sizes

^a Diapausing versus directly developing (1st and 2nd directly developing generation pooled) individuals

^b Wild caught (4 years data pooled) versus lab-reared adults (2009 larval growth experiment)

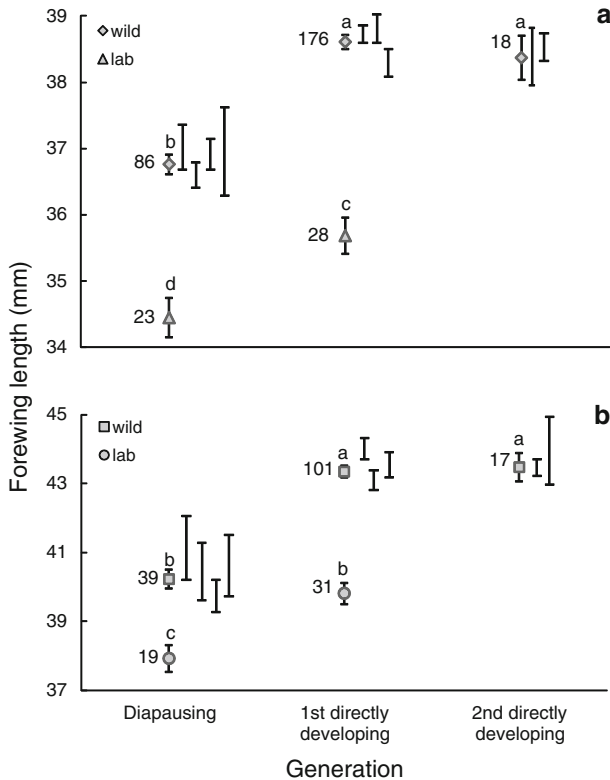


Fig. 1 Body sizes of *I. podalirius* adults representing different generations. Symbols indicate mean (± 1 SE) forewing lengths of wild-caught **a** male and **b** female adults compared to laboratory-reared individuals from the 2009 larval growth experiment. Letters indicate significant differences based on Tukey–Kramer post hoc multiple comparison tests: groups marked with the same letter are not significantly different at the $P = 0.05$ level. Numbers stand for sample sizes. Plain error bars indicate within-year (1998–2001) variation in wing lengths of wild-caught individuals (mean ± 1 SE)

comparison tests, only the difference between spring trial females and summer trial females retained significance (Fig. 2). Larval development time was significantly shorter (20 %, on average) in the spring trial than in the summer and autumn trials (LMM: $F_{2,43} = 26.5$, $P < 0.001$; MCMC model: $P < 0.001$, $\Delta DIC = 14.8$; Fig. 2). In both years larval mortality was lower in the spring than in the summer/autumn trials (8 and 34 %, respectively for 2008 and 70 and 87 % for 2009).

Larval growth experiment: final size and larval development time

In both years, all individuals in the long day treatment developed directly while the short day treatment always induced diapause. Long day treatment individuals attained significantly higher (5–10 %) pupal weights than those reared under the short day treatment (Table 4; Figs. 3, 4). The differences between the photoperiodic treatments were even larger for adult weight (only data for the 2009 experiment were available): in both sexes long day treatment individuals were approximately 20 % heavier (LM with sex in the

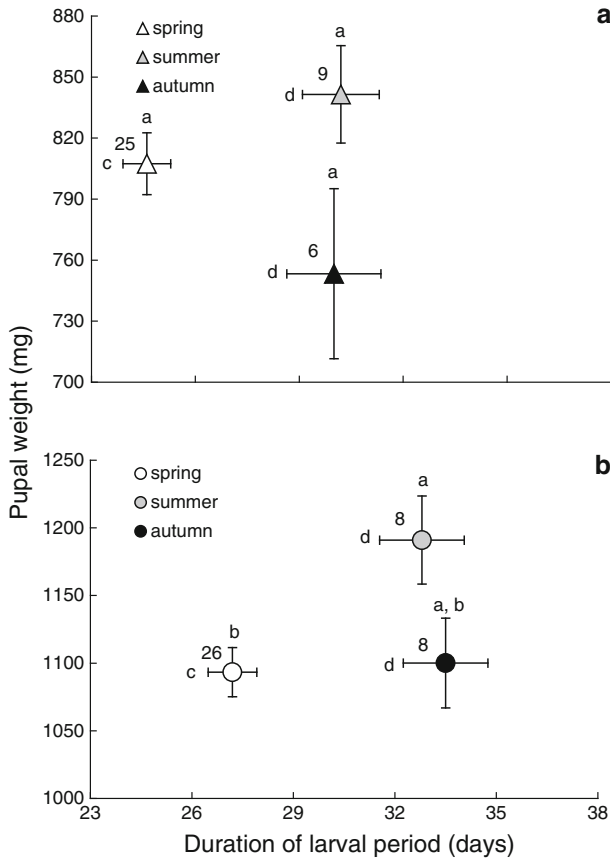


Fig. 2 Mean (± 1 SE) pupal weights and durations of larval periods in the food quality experiment for **a** males and **b** females, all reared under long day conditions. Values are corrected for the effect of brood (nested within trial using SAS, PROC MIXED, least square means option, Littell et al. 2006). Letters indicate significant differences based on Tukey–Kramer post hoc multiple comparison tests (*a–b* for pupal weight, *c–d* for duration of larval period). Groups marked with the *same* letter are not significantly different at the $P = 0.05$ level. Numbers indicate sample sizes

model: $F_{1,104} = 41.2$, $P < 0.001$) than those reared under the short day conditions. Consistently, weight loss during the pupal stage (pupal weight/adult weight) was significantly higher for short day treatment individuals ($F_{1,104} = 25.3$, $P < 0.001$). Pupal weight was highly correlated with adult weight (linear regression with sex in the model, $R^2 = 82\%$, $P < 0.001$, $n = 107$) and with adult forewing length ($R^2 = 75\%$, $P < 0.001$, $n = 99$). There were no significant differences in the larval development time between the photoperiodic treatments (LM with sex and year in the model: $F_{1,203} = 0.7$, $P = 0.4$).

Larval growth experiment: younger instars

No differences in size between long and short day treatment larvae were noted by the end of the 4th (penultimate) instar (Fig. 3). Curiously, male larvae developing under short day conditions were even significantly larger than long day treatment males at the beginning of 4th instar (Fig. 3a). Neither were there any significant differences between photoperiodic

Table 4 Comparison of growth parameters (mean \pm SE) of the final (5th) instar *I. podalirius* larvae in larval growth experiment

Years	Treatment	n ^a	Weight		Duration (days)			Growth rates ^b	
			Premoult	Maximal	Pupal	Entire instar	Positive growth phase ^c	Negative growth phase ^d	Instantaneous ^e Positive growth phase
Years pooled	LD ^f	104	284.1 \pm 3.1 (ns) ^h	–	798.3 \pm 7.2***	13.8 \pm 0.2* ⁱ	–	–	0.54 \pm 0.02 (ns)
	SD ^g	114	283.7 \pm 3.1	–	739.5 \pm 7.1	14.3 \pm 0.5	–	–	0.56 \pm 0.02
2008	LD	28	275.7 \pm 7.3 (ns)	–	783.9 \pm 21.5*	13.4 \pm 0.5**	–	–	0.55 \pm 0.07 (ns)
	SD	26	268.6 \pm 7.2	–	718.2 \pm 21.2	14.8 \pm 0.5	–	–	0.54 \pm 0.07
2009	LD	76	281.6 \pm 3.5 (ns)	1,141.0 \pm 12.5**	806.5 \pm 7.7***	14.3 \pm 0.3 (ns)	9.6 \pm 0.2 ⁺	4.7 \pm 0.1***	0.54 \pm 0.02 (ns)
	SD	88	284.1 \pm 3.4	1,084.0 \pm 12.3	747.4 \pm 7.6	14.5 \pm 0.3	9.1 \pm 0.2	5.4 \pm 0.1	0.57 \pm 0.02

All values are corrected for sex (ANOVA, least square means), 2008 trial values are corrected for brood and years-pooled dataset is corrected for year

^a Number of successfully pupated individuals

^b Calculated as: (weight at the end of the phase 1/3—weight at the end of the previous phase 1/3)/duration of the phase

^c From moult to achievement of maximal body weight

^d From achievement of maximal body weight to pupation

^e Second day of the instar

^f Long day (18L:6D), inducing directly developing generation

^g Short day (12L:12D), inducing diapausing generation

^h Significance level (long day treatment compared with the short day treatment): not significant (ns), $P > 0.1$; * $P < 0.1$; ** $P < 0.05$; *** $P < 0.001$

ⁱ Year*treatment interaction had significant effect in the model

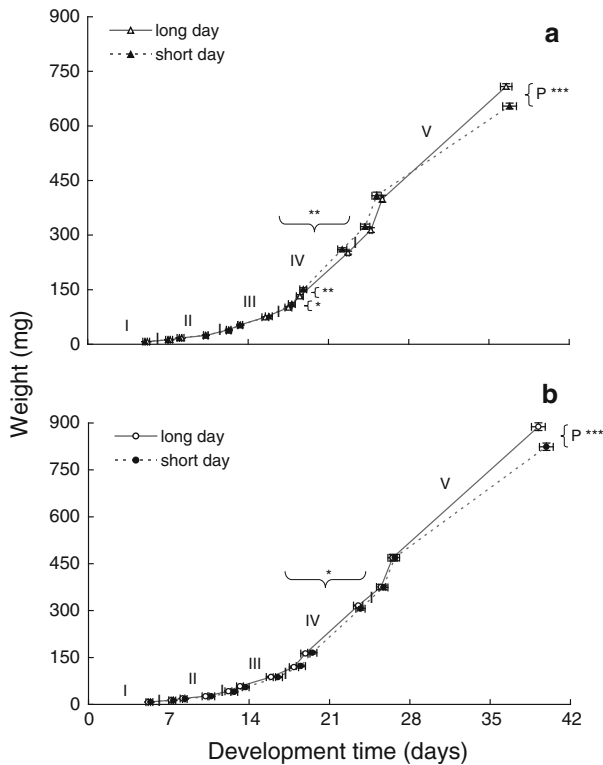


Fig. 3 Growth curves of **a** male and **b** female larvae in the two photoperiodic treatments, as based on mean weights (± 1 SE) on the second, third and final day of each instar and on mean durations of instars (± 1 SE). Results of the 2008 and 2009 larval growth experiments have been pooled. Vertical lines indicate moults, Roman numerals stand for instars and ‘P’ indicates pupation. Significant differences between the photoperiodic treatments in weights of particular days and durations of instars are marked with asterisks. See Table 4 for statistical details

treatments in the total duration of the four youngest instars (LM with sex and year in the model: $F_{1,203} = 0.04$, $P = 0.8$). Consistently, durations of particular instars did not differ between photoperiodic treatments for instars 1–3 (Fig. 3). However, 4th instar was significantly longer (though just 7 %) in the long day treatment ($F_{1,204} = 12.35$, $P = 0.005$; Fig. 3).

Larval growth experiment: last instar

Not only were the larvae of equal sizes at the beginning of their 5th instar, but the difference between photoperiodic treatments did not appear until the very last days of the final instar (Table 4; Fig. 4). In particular, long day treatment larvae were first recorded to be heavier than the individuals in the short day treatment only after the latter had started to lose weight by the end of the last instar (i.e. after the beginning of the negative growth phase, Table 4; Fig. 4). Moreover, a more detailed comparison of positive growth phases of the last instar (a non-linear mixed model with a three-parameter logistic function, see “Materials and methods” section) indicated no significant differences in growth between the two photoperiodic treatments during that period ($F_{3,1110} = 0.6$, $P = 0.6$).

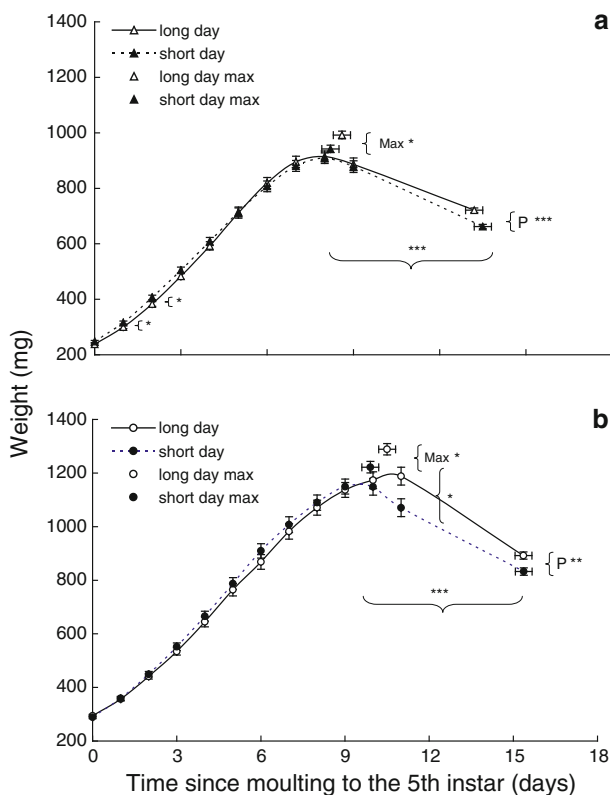


Fig. 4 Growth curves (mean weight of particular day ± 1 SE) of **a** male and **b** female 5th instar larvae in two photoperiodic treatments (experiment of 2009). ‘Max’ indicates the maximum weight of the larvae and ‘P’ stands for pupation. Significant differences between the photoperiodic treatments are marked with asterisks. See Table 4 for statistical details

Short day treatment larvae had significantly longer 5th instars than larvae from the long day treatment in the years-pooled dataset in 2008, but not in the 2009 experiment (Table 4). Inconsistencies between the years were also indicated by a significant year*treatment interaction term. The difference in the duration of the positive growth phase of the last instar was not significant between photoperiodic treatments. However, the negative growth phase (from the attaining of maximal body weight to pupation) was significantly (15 %) longer in short than in long day treatment larvae (Table 4). Growth rates did not differ between the photoperiodic treatments either when calculated over the positive growth phase of the instar or when instantaneous growth rates of the second day of the instar were compared (Table 4).

Discussion

Our field data on forewing lengths of the wild-caught *I. podalirius* individuals confirmed that adult sizes of the directly developing individuals are indeed consistently larger than in the diapausing ones (Fig. 1; Table 2). The simplest explanation of this pattern could be

based on responsive plasticity, i.e. immediate effects of seasonally varying environmental variables on larval growth schedules. However, as discussed below, this appears unlikely. Instead, between-generation size differences seem to be attributable to anticipatory plasticity (i.e. induced in response to environmental cues) in larval growth schedules.

In particular, our results do not support the possibility that seasonal differences in growth trajectories leading to between-generation size differences are caused by a seasonal change in host plant quality (e.g. Hahn and Denlinger 2007). Phenological stage of the host plant had an influence on pupal weights, but only in one sex and not in the expected direction. More specifically, early season female larvae, despite having received food of presumably highest quality, matured at even smaller size than those reared on lower quality food in summer (Fig. 2). Nevertheless, the duration of larval period was approximately 20 % shorter in the spring than in later on the season (Fig. 2), indicating that host quality was higher after all (c.f. Gebhardt and Stearns 1988, 1993; Tammaru 1998; Esperk and Tammaru 2010). The higher quality of spring leaves is also supported by literature data (e.g. Schroeder 1986 for *Prunus serotina*) and, in the current study, by the higher larval mortality in the late-season trials.

There are also several lines of evidence against the possibility that seasonal differences in growth schedules in *I. podalirius* are caused by seasonal variation in ambient temperature. In particular, for temperature to be the primary factor underlying the between-generation size differences in *I. podalirius*, the following should be expected. First, there is typically high variation in ambient temperatures between the years (AEMET 2011a). A much more irregular pattern of body sizes than actually recorded (Fig. 1) should be the consequence. Second, daily average temperatures in the study area are more than 3 °C higher at the time of the larval period of the 2nd than that of the 1st directly developing generation (June–July vs. May–June; AEMET 2011b). As higher temperatures typically lead to smaller body sizes in insects (Atkinson 1994), individuals representing the 2nd directly developing generation should be smaller. Third, in natural populations of *I. podalirius*, part of the diapausing generation is the progeny of the 1st directly developing generation while another part is offspring of the 2nd directly developing generation (Table 1). During their larval period these groups are subjected to an average temperature difference of 4 °C (July–August vs. September–October AEMET 2011b), which should translate into higher variation of adult size of the diapausing generation compared to the directly developing generation. However, none of these three expectations was supported by our results (see Tables 2, 3; Fig. 1).

The conclusion that the between-generation differences in growth schedules are attributable to anticipatory rather than responsive plasticity is further supported by our finding that directly developing individuals also grow larger under controlled laboratory conditions. As the only difference between our controlled simultaneous treatments was in the photoperiod, a typical cue used in adaptive decision-making in insects (e.g. Tauber et al. 1986), it appears straightforward to interpret the observed differences as representing anticipatory plasticity. Indeed, as the larvae of *I. podalirius* do not appear to have notable circadian differences in feeding activity, the immediate effect of photoperiod does not appear as a likely alternative.

Perhaps the strongest argument against an immediate effect of photoperiod on growth schedules is the appearance of the size differences between the diapausing and directly developing individuals only very late in the last instar, i.e. at the time when the diapausing individuals had already stopped growing and started to lose weight (Fig. 4). Indeed, before that point the directly developing and diapausing individuals showed no differences in growth rates and durations of developmental phases. An immediate environmental effect

could hardly cause this type of divergence in growth schedules as instantaneous growth rate is the parameter most likely to be affected under a responsive plasticity scenario.

As the evidence is strongly biased in favour of anticipatory rather than responsive plasticity, it is likely that seasonal differences in growth trajectories have an adaptive basis (c.f. Shapiro 1976; Brakefield and Frankino 2009). However, it remains to be tested whether size polyphenism may be adaptive *per se* (i.e. if the relationship between size and fitness differs seasonally), something which has been suggested for some other butterflies (Friberg and Karlsson 2010; Gotthard and Berger 2010). Nevertheless, the most likely ultimate reason for the between-generation differences in larval growth trajectories of *I. podalirius* appears to be related to the presence or absence of the overwintering stage in the future development of the insect. The crucial role of hibernation as such is supported by the fact that the two non-diapausing generations, both lacking overwintering stage, did not differ from each other in size while the diapausing one was an obvious outlier (Fig. 1). Indeed, surviving the adverse winter conditions necessarily requires specific adaptations (Košťál 2006; Hahn and Denlinger 2007, 2011). In insects, initiation of diapause is preceded by a preparation phase and is often characterized by specific physiological processes, such as intensive accumulation of energetic reserves and cryoprotectants, and also by specialized behavioural patterns, like active search of and movement to localities suitable for overwintering (Tauber et al. 1986; Leather et al. 1993; Hahn and Denlinger 2007, 2011).

In *I. podalirius*, the exact nature of the physiological differences between overwintering and directly developing generations remains yet to be studied. Nevertheless, there are considerable between-generation differences in prepupal behaviour of the larvae. While directly developing larvae normally pupate on the host plants, larvae of the diapausing generation move away from the host to pupate in the leaf litter (Table 1), most likely to avoid bird predation in winter (Stefanescu 2004). As a consequence, the time spent in the prepupal “wandering” phase (Dominick and Truman 1984) of latter group is notably longer (Fig. 4; Stefanescu unpublished). Due to these behavioural differences, diapausing individuals are likely to lose more energy and body weight already prior to entering the diapauses, in addition to the perhaps inevitably higher energetic losses during the much longer pupal period itself (Scriber 1994; Friberg and Karlsson 2010; Friberg et al. 2011).

Taking it together, this study suggests that a ‘simple’ explanation for between-generation size difference as based on responsive plasticity is unlikely even for the species in which the individuals of the overwintering generation are of a smaller size. In particular, an adaptive character of the plastic difference appears intuitive in those species with overwintering individuals being larger than directly developing ones (Teder et al. 2010). However, it is rather straightforward to ascribe the small size of the individuals developing at the end of the season to an immediate effect of the suboptimal conditions they are faced with (e.g. Scriber and Slansky 1981; Van Asch and Visser 2007). This study implies that this is not necessarily the case and therefore indicates that the adaptive nature of the seasonal polyphenism in body size in insects may have a rather universal character. The question to be posed by forthcoming studies is thus whether there are any examples of regular among-generation differences which are primarily based on responsive plasticity. An aspect to be emphasized is the value of detailed monitoring of individual development because, as has been shown here, knowing when and how in the ontogeny the differences appear may provide relevant information. In particular, the fact that the among-generation differences in *I. podalirius* in body weight appear after the larvae have ceased feeding strongly points at an ‘adaptive decision’ rather than at an immediate effect of growing conditions (see Tammaru et al. 1999, for an analogous example on the duration of pupal

period). Support for the adaptive character of a plastic change may thus be possible even when obtaining the most direct evidence, i.e. measuring fitness of different phenotypes in different conditions, is too complicated for practical reasons, as is the case for the highly mobile adults of many butterfly species.

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